

# Sirtuin-1 Targeting Promotes Foxp3<sup>+</sup> T-Regulatory Cell Function and Prolongs Allograft Survival<sup>▽</sup>

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**Sirtuin 1 (Sirt1), a class III histone/protein deacetylase, is central to cellular metabolism, stress responses, and aging, but its contributions to various host immune functions have been little investigated. To study the role of Sirt1 in T cell functions, we undertook targeted deletions by mating mice with a floxed Sirt1 gene to mice expressing CD4-cre or Foxp3-cre recombinase, respectively. We found that Sirt1 deletion left conventional T-effector cell activation, proliferation, and cytokine production largely unaltered. However, Sirt1 targeting promoted the expression of Foxp3, a key transcription factor in T-regulatory (Treg) cells, and increased Treg suppressive functions *in vitro* and *in vivo*. Consistent with these data, mice with targeted deletions of Sirt1 in either CD4<sup>+</sup> T cells or Foxp3<sup>+</sup> Treg cells exhibited prolonged survival of major histocompatibility complex (MHC)-mismatched cardiac allografts. Allografts in Sirt1-targeted recipients showed long-term preservation of myocardial histology and infiltration by Foxp3<sup>+</sup> Treg cells. Comparable results were seen in wild-type allograft recipients treated with Sirt1 inhibitors, such as EX-527 and splitomicin. Hence, Sirt1 may inhibit Treg functions, and its targeting may have therapeutic value in autoimmunity and transplantation.**

Sirtuins are NAD<sup>+</sup>-dependent class III histone/protein deacetylase (HDAC) enzymes that are highly conserved across eukaryotic species (7). Sirtuins regulate multiple important processes in cell physiology by catalyzing  $\epsilon$ -acetyl-lysine deacetylation (29). Such deacetylation promotes gene silencing by chromatin condensation and also modifies the stability and functions of various nonhistone proteins. Sirtuin 1 (Sirt1) is a key factor in the caloric restriction response and life span control and is also a therapeutic target in various cancers (13, 29). Consequently, investigators have sought, respectively, to either induce (14) or suppress (10) its functions. Currently, relatively little is known of the role of sirtuins in immune responses, though outbred mice with a global knockout of Sirt1 show failure to thrive, have shortened life spans, and display autoimmune-prone phenotypes (24, 34).

Conventional Zn-dependent class I and II HDACs are under investigation as targets to suppress immune responses (30). While HDACs play important roles in multiple immune cell types, recent studies have focused on the ability of conventional HDACs to deacetylate forkhead box P3 (Foxp3), a key transcription factor in T-regulatory cells (Tregs), and accelerate disassembly of multicomponent Foxp3 complexes (26). Thus, certain Zn-dependent HDAC enzymes impair, whereas corresponding HDAC inhibitors enhance, Treg function (30). These data led us to hypothesize that sirtuins might play important roles in conventional T-effector and/or Treg cell biology. Given that the bulk of data concerning sirtuins involves

Sirt1 and that both Sirt1 null mice and Sirt1 inhibitors have been reported, we focused on this specific sirtuin. Global deletion of Sirt1 may have developmental effects on immune functions, and findings may be further affected by use of mice on an outbred background. We therefore deleted Sirt1 in conventional T cells, or specifically in Tregs, by mating standard inbred C57BL/6 mice bearing a floxed Sirt1 (fl-Sirt1) gene (3) to mice expressing CD4-cre (11) or Foxp3-cre recombinase (20), respectively. We assessed the effect of transient Sirt1 inhibition in wild-type (WT) mice using the commercially available small-molecule inhibitors splitomicin (16), a general sirtuin inhibitor, and EX-527, a specific Sirt1 inhibitor (25). Based on the resultant data, we also characterized the role of Sirt1 deletion and inhibition on T cell function and allograft survival *in vivo*.

## MATERIALS AND METHODS

**Mice and cardiac allografting.** BALB/c (H-2<sup>d</sup>), C57BL/6 (H-2<sup>b</sup>), Thy1.1 (H-2<sup>b</sup>), B6/RAG1<sup>-/-</sup> (H-2<sup>b</sup>), C57BL/6/DBA2 (H-2<sup>b/d</sup>) (The Jackson Laboratory) and fl-Sirt1/CD4cre and fl-Sirt1/Foxp3cre (H-2<sup>b</sup>) mice were housed under specific-pathogen-free conditions and studied using a protocol approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia. We transplanted BALB/c hearts into the abdomens of fl-Sirt1/CD4cre, fl-Sirt1/Foxp3cre, B6/Rag1<sup>-/-</sup>, and wild type (WT) C57BL/6 mice (26). Recipients were untreated or received splitomicin or EX-527 (1 mg/kg of body weight/day) plus rapamycin (0.1 to 0.2 mg/kg/day) intraperitoneally (i.p.) for 14 days from engraftment. Allograft survival was assessed by daily palpation, and rejection was confirmed by histology.

**Cell isolation and flow cytometry.** Spleen and lymph nodes were processed to single-cell suspensions and separated into antigen-presenting cells (APC) (Thy1.2<sup>-</sup>), T-effector cells (CD4<sup>+</sup> CD25<sup>-</sup>), and Tregs (CD4<sup>+</sup> CD25<sup>+</sup>) using magnetic beads (Miltenyi Biotec). Cells of interest were analyzed using surface markers, and for Foxp3 staining, surface marker-stained cells were fixed, permeabilized, and labeled with Foxp3-specific monoclonal antibody (MAb) (27). For cytokine production, T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (3 ng/ml) plus ionomycin (1  $\mu$ M) for 6 h. Data were analyzed using the Flowjo 6.4.7 (Treestar) software program.

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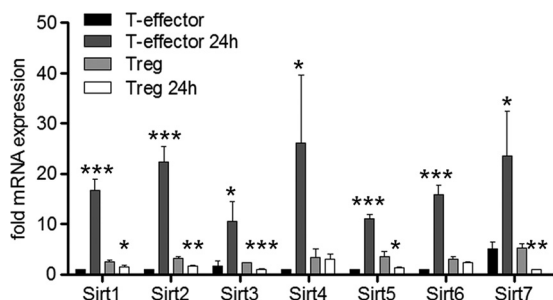


FIG. 1. Differential effects of cell activation on sirtuin gene expression by conventional T cells versus Tregs. Expression of sirtuin genes 1 to 7 (qPCR, mean  $\pm$  SD, 3/group) by resting and activated T-effector cells and Tregs is shown. Activation was induced for 24 h using CD3 $\epsilon$  MAb (1  $\mu$ g/ml) and irradiated APC, and data from stimulated versus unstimulated cells ( $n$  = 3/group) were assessed (\*,  $P$  < 0.05; \*\*,  $P$  < 0.01; \*\*\*,  $P$  < 0.001 [versus results for unstimulated cells]).

**T-effector cell functions.** Purified T-effector cells were stimulated with irradiated APC plus CD3 $\epsilon$  with or without CD28 MAb (1  $\mu$ g/ml). After 72 h, cells were labeled with CD4 fluorescent MAb and analyzed by flow cytometry. For parent-to-F1 assays, we injected  $4 \times 10^7$  carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes)-labeled T-effector cells into C57BL/6/DBA2 mice, and at 1 week, splenocytes were analyzed by flow cytometry. For conversion to Foxp3 $^{+}$  Tregs, T-effector cells were incubated with transforming growth factor  $\beta$  (TGF- $\beta$ ) (3 ng/ml) and interleukin 2 (IL-2) (25 U/ml) for 3 days, and the amount of cells that converted positive to Foxp3 $^{+}$  was assessed by flow cytometry (5).

**In vitro and in vivo Treg suppression assays.** CFSE-labeled WT T-effector cells, with or without Tregs, were incubated for 72 h in the presence of irradiated WT APC and CD3 $\epsilon$  MAb, labeled with CD4-fluorescent MAb and analyzed by flow cytometry (26). To assess several independent Treg suppression assays quantitatively, we subtracted cell proliferation rates of T-effector cells treated with Sirt1 $^{-/-}$  Tregs from rates of those treated with WT Tregs and tested if the difference was significant to null. For *in vivo* Treg suppression assays, we adoptively transferred  $1 \times 10^6$  Thy1.1 $^{+}$  CD4 $^{+}$  T-effector cells without or without  $2.5 \times 10^5$  Tregs into B6/RAG1 $^{-/-}$  mice (26). Lymphocytes were isolated 1 week later, and expansion of Thy1.1 $^{+}$  CD4 $^{+}$  cell populations was assessed by flow cytometry.

**RNA isolation, reverse transcription, and quantitative PCR.** RNA extracted using RNeasy kits (Qiagen) were reverse transcribed to cDNA with random hexamers and amplified (PTC-200; MJ Research). Primer and probe sequences for target genes were used for quantitative PCR amplification of total cDNA (StepOnePlus real-time PCR system and TaqMan assay reagents; Applied Biosystems). Relative quantitation of target cDNA was determined by setting the control value to 1, and changes in cDNA content were expressed as fold increases above the set control value. Differences in cDNA input were corrected by normalizing signals obtained with specific primers for 18S rRNA, and nonspecific amplification was excluded by performing quantitative PCR in the absence of target cDNA.

**Immunoprecipitation and Western blotting.** We precleared proteins extracted from Tregs with protein G and incubated them overnight with anti-Foxp3 MAb (eBioscience), followed by immunoprecipitation with protein G, elution, and Western blotting (25). For direct immunoprecipitation, we covalently bound anti-Foxp3 MAb to magnetic Dynabeads (Invitrogen) overnight at 37°C. Next, we magnetically separated CD4 $^{+}$  CD25 $^{+}$  Tregs as above and isolated protein from Tregs according to the manufacturer's instructions. Subsequently, the lysate was incubated with the anti-Foxp3-coupled Dynabeads for 60 min at 4°C, followed by elution of the Dynabeads and Western blotting.

**Pathology.** Paraffin sections of cardiac allografts were stained with hematoxylin and eosin (H&E). Cryostat sections of corresponding snap-frozen tissues were fixed with 2% paraformaldehyde, rinsed in buffer, pretreated with 10% normal goat serum and 0.1% Triton X-100 (Sigma-Aldrich), and incubated overnight with rat MAb to CD4 (H129.19, 5  $\mu$ g/ml; BD Pharmingen), CD8 (53-6.7, 5  $\mu$ g/ml; BD Pharmingen), CD45 (30-F11, 5  $\mu$ g/ml; BD Pharmingen), and Foxp3 (FJK-16S, 5  $\mu$ g/ml; eBioscience) diluted in 2% normal goat serum and 0.1% Triton X-100. Sections were washed and incubated with rabbit anti-rat IgG (Dako) plus H $_2$ O $_2$  in methanol (4°C, 10 min) to block endogenous peroxidases, and bound antibodies were detected using peroxidase-conjugated anti-rabbit IgG and diaminobenzidine substrate (Envision kit; Dako).

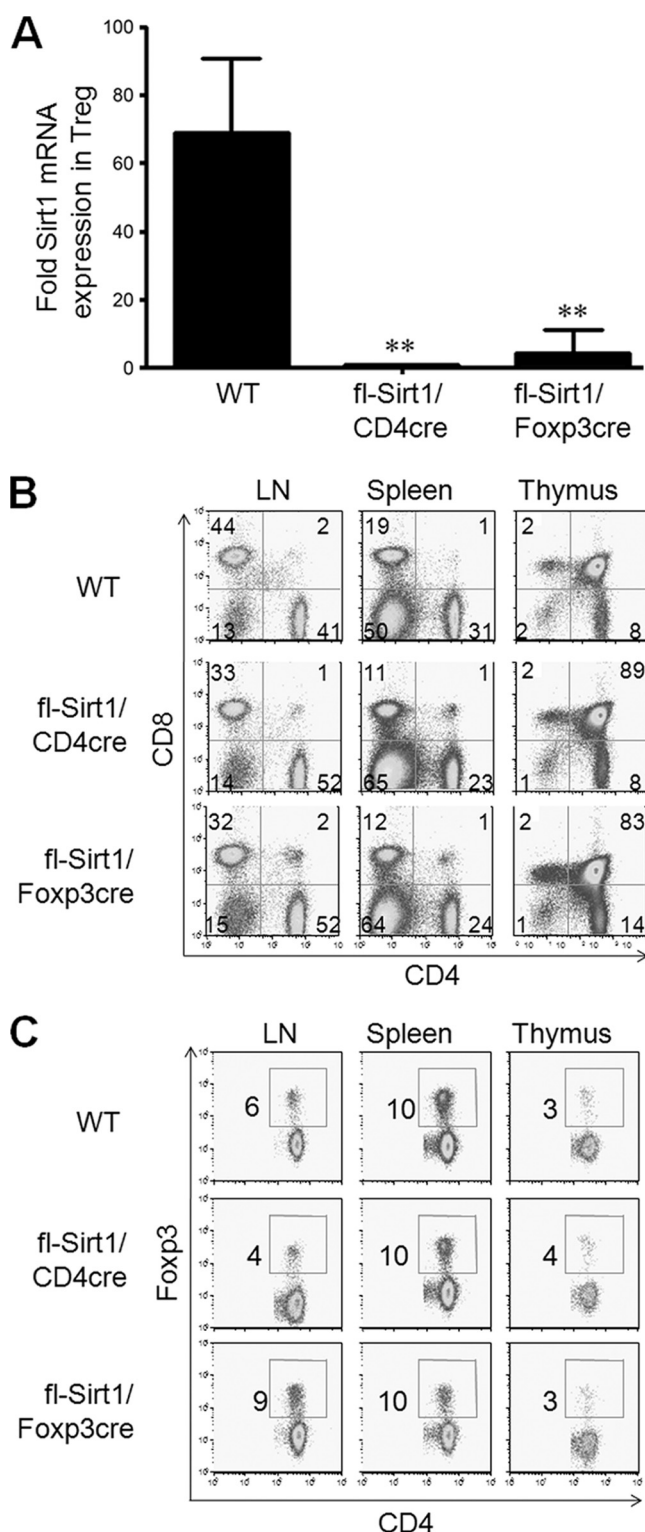


FIG. 2. Targeted deletion of Sirt1 in T cells and Tregs. (A) Expression of Sirt1 by WT Tregs (CD4 $^{+}$  CD25 $^{+}$ , isolated by magnetic beads, 85% Foxp3 $^{+}$  Treg purity) versus corresponding cells from fl-Sirt1/CD4cre or fl-Sirt1/Foxp3cre mice (qPCR, mean  $\pm$  SD, 3/group; \*\*,  $P$  < 0.01 versus results for WT). (B and C) Flow cytometric analysis of T cell populations in thymi, peripheral lymph nodes (LN), and spleens of WT mice or mice with deletion of Sirt1 using CD4cre (B) or Foxp3cre (C); the percentage of each population is indicated, and data are representative of 3 mice/group.

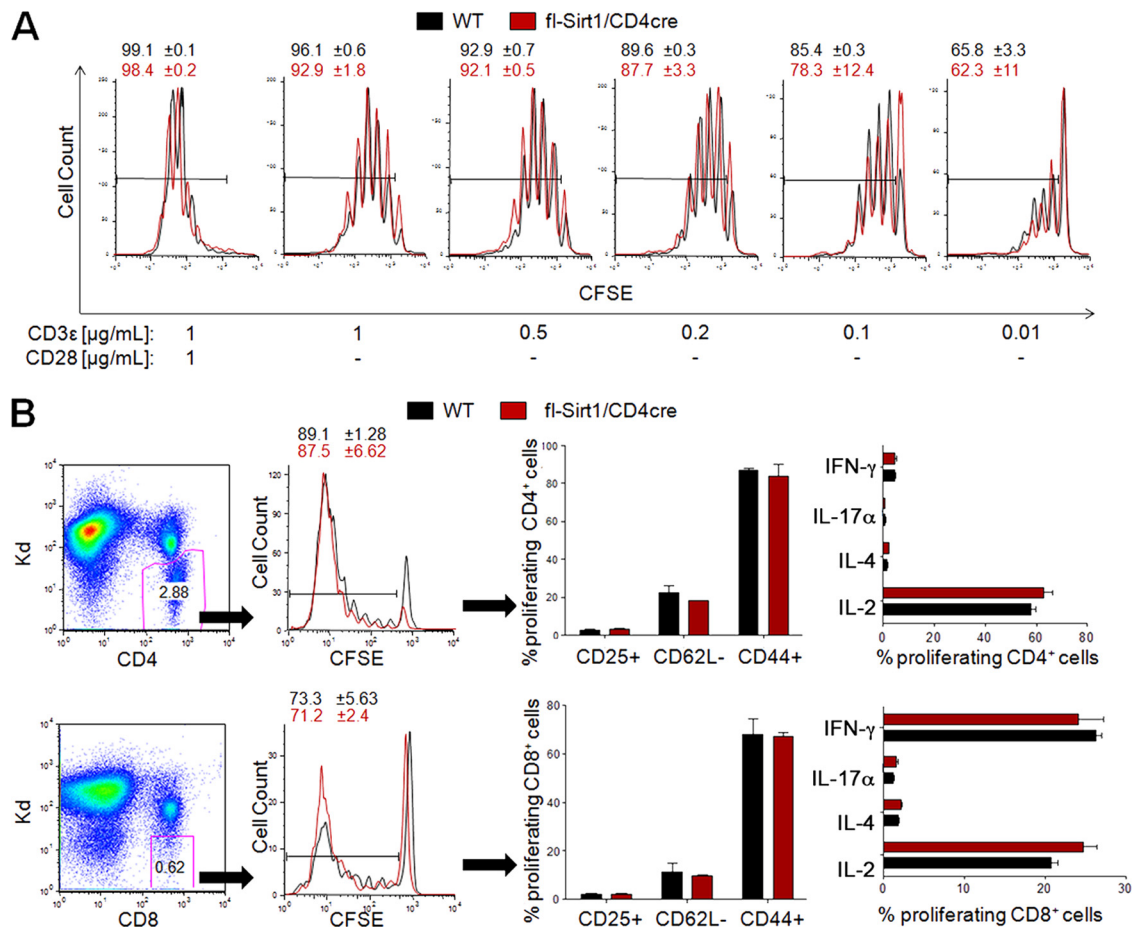


FIG. 3. Sirt1 deletion does not alter T-effector cell activation. (A) *In vitro* stimulation of CFSE-labeled CD4<sup>+</sup> CD25<sup>-</sup> T-effector cells showed no difference in proliferation between fl-Sirt1/CD4cre and WT cells upon activation by CD3ε ± CD28 MAb and APC ( $1 \times 10^6$ /ml). (B) Parent-to-F1 assay: CFSE-labeled T-effector cells from WT or fl-Sirt1/CD4cre mice (both H-2<sup>b</sup>) were injected into H-2<sup>d</sup> mice. After 3 days, injected cells (H-2<sup>d</sup> negative) were analyzed for proliferation, cellular activation, and cytokine production. For all parameters shown, data from fl-Sirt1/CD4cre and WT T-effector cells were comparable; error bars represent results of 3 independent assays ( $P > 0.05$  in all cases).

**Microarrays.** RNA integrity and quantity were analyzed by photometry (DU640; Beckman-Coulter). Microarray experiments were performed using whole-mouse-genome oligoarrays (Mouse430a; Affymetrix), and array data were analyzed using the MAYDAY 2.10 software program (1). Array data were subjected to robust multiarray average (RMA) normalization. Normalized data were used for calculating fold changes of up- and downregulated genes using Student's *t* test. Only data with a false discovery rate (FDR)-adjusted *P* value of  $<0.05$  and at least 2× differential expression were included in the analysis. Data underwent z-score transformation for display.

**Statistical analysis.** Data were analyzed using the GraphPad Prism 5.01 and Stata 10 software programs. All normally distributed data were displayed as means ± standard deviations (SD). Measurements between two groups were done with a Student *t* test or Mann-Whitney U test. Groups of three or more were analyzed by two-way analysis of variance (ANOVA) or the Kruskal-Wallis test. Allograft survival was assessed using a log-rank (Mantel-Cox) test.

**Microarray data accession number.** We deposited our data in the NCBI Gene Expression Omnibus (GEO) database ([www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) under accession numbers GSE26425.

## RESULTS

**Contrasting effects of T cell receptor (TCR) stimulation on Sirt expression by T-effector and Treg cells.** Assessment of the expression of sirtuin genes in resting and TCR-activated T cells from WT mice showed that cell activation increased expres-

sion of all 7 sirtuins in T-effector cells (Fig. 1). However, while Tregs showed a constitutively higher level of sirtuin expression than T-effector cells, expression of 5 of 7 sirtuin genes was decreased upon TCR stimulation. Of note for our study, upon cell activation, Sirt1 was upregulated 17-fold in T-effector cells but downregulated 2-fold in Tregs compared to resting levels. These data suggested the relevance of exploring Sirt1 functions in these differing T cell populations.

### Targeted Sirt1 deletion in CD4<sup>+</sup> T cells and Foxp3<sup>+</sup> Tregs.

We mated floxed fl-Sirt1 B6 mice and CD4-cre or Foxp3-cre B6 mice and selected for fl-Sirt1/CD4cre and fl-Sirt1/Foxp3cre, respectively. Deletion of Sirt1 was confirmed by quantitative PCR (qPCR) (Fig. 2A). Despite Sirt1 deletion, fl-Sirt1/CD4cre and fl-Sirt1/Foxp3cre mice showed development of CD4<sup>+</sup>, CD8<sup>+</sup> (Fig. 2B), and Foxp3<sup>+</sup> CD4<sup>+</sup> (Fig. 2C) lymphocyte populations comparable to that of corresponding WT controls. Hence, the targeted deletion of Sirt1 did not appear to affect the corresponding development of either conventional T cells or Treg cells.

**Sirt1 deletion does not alter effector T cell function.** Since fl-Sirt1/CD4cre mice delete Sirt1 in both effector T cells and

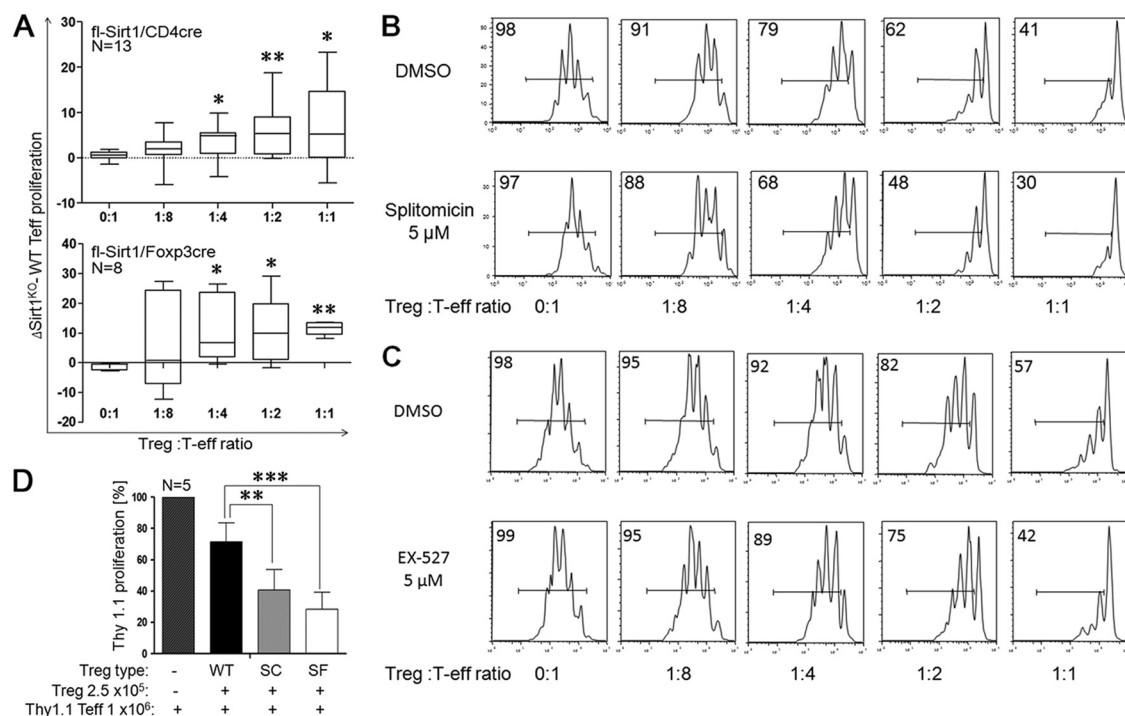


FIG. 4. Sirt1 and Treg function. (A) Comparison of the ability of Sirt1<sup>-/-</sup> versus WT Tregs to suppress proliferation of CFSE-labeled WT effector T cells *in vitro*, showing the enhanced suppressive function of Sirt1<sup>-/-</sup> Tregs (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$  [versus results for WT Tregs]). (B) *In vitro* suppression assay showing effect of the sirtuin inhibitor splitomycin on WT Treg function; residual proliferation of CFSE-labeled T cells is shown in each panel. (C) *In vitro* suppression assay showing effect of the Sirt1-specific inhibitor EX-527 on Treg function; residual proliferation of CFSE-labeled T cells is shown in each panel. (D) Homeostatic proliferation showed enhanced suppressive function of Sirt1 deletion Tregs *in vivo* (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ ). Abbreviations: SC, fl-Sirt1/CD4cre; SF, fl-Sirt1/Foxp3cre.

Tregs, we assessed proliferation, activation, and cytokine production by CD4<sup>+</sup> CD25<sup>-</sup> effector T cells from fl-Sirt1/CD4cre mice versus data for WT mice. We found that proliferation in response to *in vitro* stimulation of T-effector cells was comparable for WT and Sirt1-deficient T cells (Fig. 3A). To assess the effects of Sirt1 deletion on the alloactivation, proliferation, and cytokine production of T-effector cells *in vivo*, we undertook parent-to-F1 injection of either fl-Sirt1/CD4cre or WT B6 T-effector cells into B6/DBA mice. We found that both populations had virtually identical cell activation, proliferation, and cytokine production after 3 days *in vivo* (Fig. 3B). Likewise, *in vitro* TCR-induced cytokine mRNA production was comparable between WT and Sirt1-deficient T cells (data not shown). We also questioned whether a lack of Sirt1 rendered T cells more likely to convert to Tregs and produce a more tolerant phenotype, but we found that TGF- $\beta$ -driven *in vitro* conversion was unaffected by Sirt1 deletion, and mesenteric lymph nodes exhibited a normal population of Foxp3<sup>+</sup> Tregs (data not shown). These data indicate that Sirt1 did not influence key responses of T-effector cells to activation under various conditions *in vitro* or *in vivo*.

**Sirt1 targeting improves Treg suppressive function.** In contrast to a lack of obvious effects of Sirt1 deletion on the TCR-driven responses of conventional T cells, Sirt1 targeting by fl-Sirt1/CD4cre or fl-Sirt1/Foxp3cre mice increased Treg suppressive function *in vitro* (Fig. 4A). These findings were reproduced using WT Tregs treated with small-molecule inhibitors of Sirt1, splitomycin (Fig. 4B), and EX-527 (Fig. 4C). To con-

firm our findings *in vivo*, we adoptively transferred T-effector cells from Thy1.1 mice and Tregs from WT, fl-Sirt1/CD4cre, or fl-Sirt1/Foxp3cre mice into immunodeficient B6/RAG-1 mice at a 4:1 ratio. Consistent with our *in vitro* findings, Sirt1-deficient Tregs showed a more potent suppressive capacity than WT Tregs (Fig. 4D). Hence, genetic or pharmacologic targeting of Sirt1 enhances Treg suppressive activity *in vitro* and *in vivo*.

**Sirt1 deletion promotes expression of Foxp3 and other genes in Tregs.** Consistent with the increased suppressive function of Tregs lacking Sirt1, qPCR studies showed Sirt1-deficient Tregs had increased mRNA levels of Foxp3 and other functionally competent Treg genes, including cytotoxic T-lymphocyte antigen 4 (CTLA4) and herpesvirus entry mediator (HVEM) (Fig. 5A). Increased expression of the Foxp3 protein in Sirt1<sup>-/-</sup> Tregs was confirmed by Western blotting (Fig. 5B and data not shown). Furthermore, we found that Sirt1<sup>-/-</sup> Tregs exhibited more protein of the transcription factor p65 and in particular more acetylated p65 (Fig. 5C). Microarray analysis of Tregs from WT, fl-Sirt1/CD4Cre, and fl-Sirt1/Foxp3cre mice showed that Sirt1 deletion downregulated expression of many genes typically associated with various T-effector cell populations, such as those encoding IL-4, gamma interferon (IFN- $\gamma$ ), tumor necrosis factor receptor (TNF-R) (CD120a), IL-7 receptor (IL-7R) (CD127), and IL-17 receptor A (IL-17RA) (CDw217), and upregulation of additional genes linked with Treg functions, including those encoding TGF- $\beta$  receptor 1 (TGF- $\beta$ R1) and CCR10 (21) (Fig. 6A). Sirt1 dele-

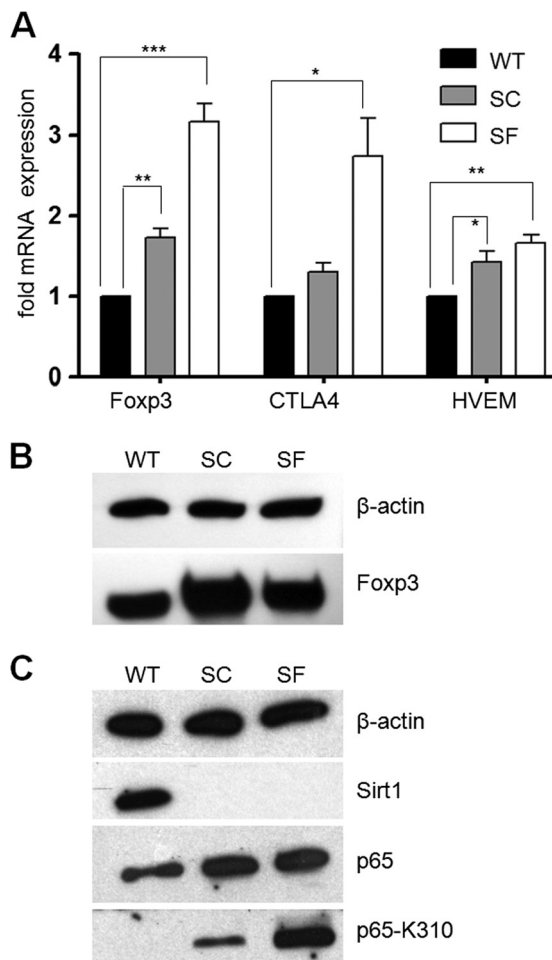


FIG. 5. Sirt1 and Foxp3 expression. (A) qPCR showing upregulation of Foxp3, CTLA-4, and herpesvirus entry mediator (HVEM) mRNA in Sirt1<sup>-/-</sup> versus WT Tregs ( $n = 4/\text{group}$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ ). (B) Western blotting showing increased Foxp3 protein in Sirt1<sup>-/-</sup> versus WT Tregs (actin loading control). (C) Immunoprecipitation of Foxp3, followed by Western blotting for acetylated lysine showed increased Foxp3 acetylation in Sirt1<sup>-/-</sup> versus WT Tregs. (C) Western blot comparing Sirt1 and p65, both total and acetylated at lysine 310, showing that Sirt1<sup>-/-</sup> Tregs exhibit more acetylated p65 and total p65. Abbreviations: SC, fl-Sirt1/CD4cre; SF, fl-Sirt1/Foxp3cre.

tion also led to upregulation of multiple genes involved in cholesterol metabolism (Fig. 6B), energy generation through the Krebs cycle (Fig. 6C), and mitochondrial electron transport pathways (not shown), as well as decreased expression of several heat shock proteins (Fig. 6D) and upregulation of HDAC1, HDAC3, and HDAC6, in addition to Sirt2 (Fig. 6E). These data indicate that Sirt1 deletion affects multiple pathways in Tregs and promotes expression of genes linked with Treg functions.

**Sirt1 neutralization prolongs allograft survival.** Last, we assessed whether the increased suppressive function of Sirt1-deficient Tregs might affect the tempo of rejection of fully major histocompatibility complex (MHC)-mismatched allografts when undertaken in conjunction with a 2-week course of a low dose of rapamycin (RPM); brief RPM therapy was em-

ployed so as to blunt T-effector cell responses in the early posttransplant period before effective Treg functions were fully developed (26). Fully MHC-mismatched cardiac allografts showed prolonged graft survival in fl-Sirt1/CD4cre mice versus WT mice (Fig. 7A). Both Sirt1 inhibitors were also effective in WT recipients when used in conjunction with the same brief course of RPM therapy, though splitomycin proved more effective than EX-527 ( $P = 0.01$ ) (Fig. 7A).

Comparison of intra-graft events around the time of rejection in control mice showed significant leukocyte infiltration (Fig. 7B) and broadly comparable CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations in allografts of both WT and Sirt1<sup>-/-</sup> recipients (Fig. 7C). However, fl-Sirt1/CD4cre recipients had much better preserved cardiac muscle architecture, with minimal myocyte encroachment or vascular injury (Fig. 7B), markedly increased numbers of intra-graft Foxp3<sup>+</sup> cells (Fig. 7C), and decreased intra-graft IFN- $\gamma$  and IL-2 effector cytokines (Fig. 7D). There were also fewer activated T cells in secondary lymphoid tissues, e.g., spleen (Fig. 7E), of Sirt1<sup>-/-</sup> recipients versus WT recipients. These findings, combined with our data on increased Treg suppressive capacity upon Sirt1 targeting, led us to hypothesize that prolonged allograft survival in Sirt1-depleted mice is chiefly driven by enhanced Treg function. To test this hypothesis, we adoptively transferred a 4:1 ratio of T-effector cells and either WT or Sirt1<sup>-/-</sup> CD4<sup>+</sup> CD25<sup>+</sup> Tregs into B6/RAG1<sup>-/-</sup> immunodeficient recipients of BALB/c cardiac allografts. Sirt1<sup>-/-</sup> Tregs proved significantly more effective than WT Tregs in promoting allograft survival under these circumstances (Fig. 7F). Likewise, use of fl-Sirt1/Foxp3 recipients plus 2 weeks of low-dose rapamycin also prolonged cardiac allograft survival compared to results for WT controls (Fig. 7G). Hence, the genetic or pharmacologic targeting of Sirt1 *in vivo* has significant beneficial effects on allograft survival, including through Treg-dependent mechanisms.

## DISCUSSION

### Selective Sirt1 targeting can attenuate immune responses.

Sirtuins are important regulators involved in nearly every system of mammalian biology (8). While their roles in the immune system have been little explored, some authors have suggested that in various cell types, Sirt1 attenuates the immune response (9, 17, 24, 34). Jung et al. observed that caloric restriction inhibits, whereas aging enhances, NF- $\kappa$ B activity (9). NF- $\kappa$ B itself, upon activation, promotes a proinflammatory state by activating transcription of cytokines such as TNF- $\alpha$  and IL-6 (12). IL-6, on the other hand, is an important factor in determining lineage commitment to Th-17 cells (36). Furthermore, mice with global deletion of Sirt1 (24, 34) or liver-specific Sirt1 deletion (17) showed autoimmune phenotypes. Most recently, Zhang et al. showed that T cells from outbred Sirt1 null mice were activated by isolated TCR signaling independently of B7-CD28 costimulation, likely via increased activator protein 1 (AP-1) activation as a result of deacetylated c-Jun (34).

In contrast, our data show enhanced Treg function and attenuated immune responses as a result of targeted Sirt1 deletion in T-effector and Treg cells. This difference likely reflects our use of targeted deletion strategies, which allow us to study the biologic effects of Sirt1 loss in specific cells. The potentially

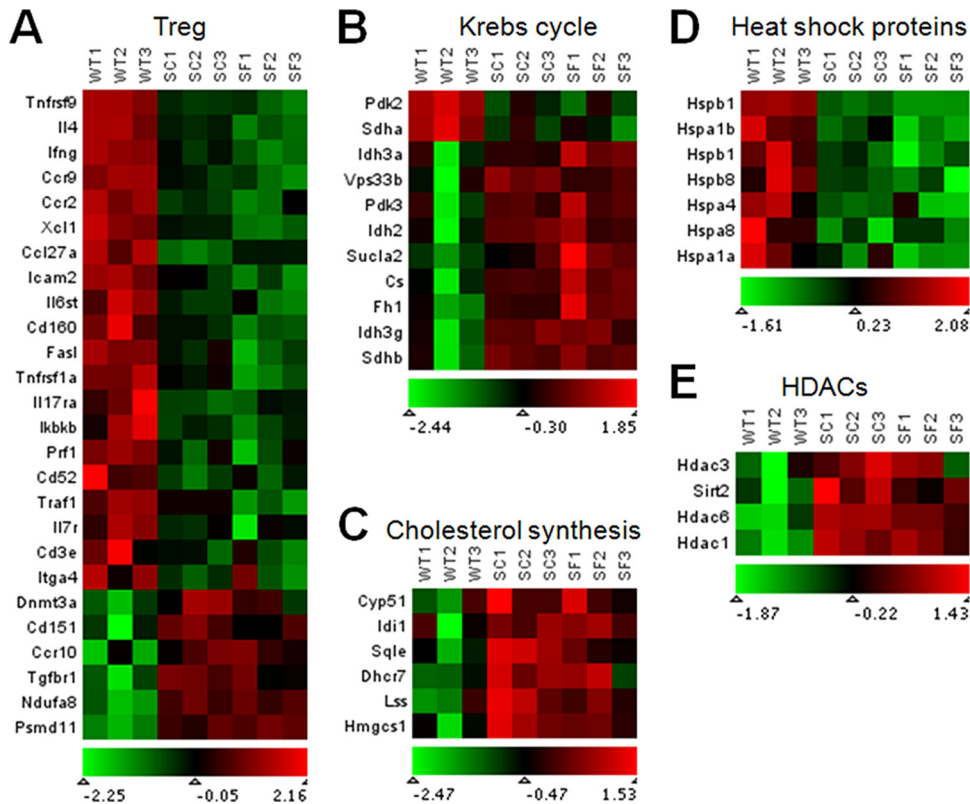


FIG. 6. Microarray analysis of expression of genes relevant to Treg function. Data displayed are  $\geq 2\times$  differentially expressed Sirt1<sup>-/-</sup> Tregs compared to WT Tregs ( $P < 0.05$ ). Data are displayed after z-score transformation; see the text for details. Abbreviations: SC, fl-Sirt1/CD4cre; SF, fl-Sirt1/Foxp3cre; Ifng, IFN- $\gamma$ ; Ccr/ccl, chemokine (C-C motif) receptor/ligand; Xcl, chemokine (C motif) ligand; Icam, intercellular adhesion molecule; Il6ST, IL-6 signal transducer; FasL, Fas ligand; Hsp, heat shock protein; Tnfrsf, tumor necrosis factor receptor superfamily; Ikbkb, inhibitor of  $\kappa$ B kinase beta; Prf, perforin; Traf, TNFR-associated factor; Itga4, integrin alpha 4; Dnmt, DNA methyltransferase; Tgfb, transforming growth factor  $\beta$  receptor; Pdk, pyruvate dehydrogenase kinase; Sdh, succinate dehydrogenase; Vps33b, vacuolar protein sorting 33B; Idh, isocitrate dehydrogenase; Sucl, succinate-coenzyme A ligase; Cs, citrate synthase; Fh, fumarate hydratase; CyP51, cytochrome P450 family 51; Idi1, isopentenyl-diphosphate delta isomerase; Hmgcs1, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1; Sqle, squalene epoxidase; Dhcr7, 7-dehydrocholesterol reductase; Lss, lanosterol synthase; Hdac, histone deacetylase.

confounding effects of removing Sirt1 from the whole organism are illustrated in Sirt1 null mice, which have a phenotype characterized by small size, infertility, and a shortened life span (8, 24, 34). It is conceivable that the complete absence of Sirt1 in the whole organism may affect the immune system throughout its development and predispose to self-reactive T cells. For example, aberrant activation of the B7-CD28 costimulation signal has been shown to bypass thymic beta selection, producing double-positive CD4/CD8 T cell precursors even in RAG1 null mice (32).

Beyond T cells, there is a growing body of literature indicating that Sirt1 suppresses activation of the inflammatory response in macrophages by several mechanisms. These include suppression of AP-1 targets, such as cyclooxygenase (35), and inhibition of LPS-induced macrophage TNF- $\alpha$  secretion (33). Recently, Schug et al. reported a murine study involving myeloid-specific Sirt1 deletion and showed that Sirt1 is crucial for deacetylating the RelA/p65 subunit of NF- $\kappa$ B. Loss of Sirt1 led to increased activation of proinflammatory cytokines (23). Indeed, our results confirm that Sirt1 is important for deacetylating RelA/p65 in Tregs as well. However, the role of RelA/p65 may be a very different one and may actually enhance the suppressive capacity of Tregs. RelA/p65 has been

proposed as part of the c-Rel enhanceosome, which is critical for the development and function of the Treg phenotype (19). Furthermore, while myeloid cells were not part of our investigation, the current data show that WT recipients of MHC-mismatched allografts treated with transient Sirt1 inhibitors show a biology similar to that of mice with targeted Sirt1 deletions in T-effector or Treg cells. Hence, while the effects of Sirt1 targeting likely vary according to the cell type under study, an important point arising from our study is that the Sirt1 targeting can achieve important therapeutic effects of T cell-dependent responses in clinically relevant experimental models. Thus, our findings offer a new insight into sirtuin biology and provide a new perspective for understanding the role of Sirt1 in the immune response.

**Mechanisms of Sirt1-depleted immune suppression.** Our data suggest that the chief mechanism enabling prolonged allograft survival through deletion of Sirt1 in T cells involves enhanced Treg function. This was documented both by prolonged allograft survival in the fl-Sirt1/Foxp3cre model with specific Sirt1 deletion in Foxp3<sup>+</sup> cells alone and by comparison of the effects of adoptive transfers of fl-Sirt1/CD4cre and WT Tregs into immunodeficient recipients of BALB/c allografts. Notably, effector T cell function was practically unaffected by

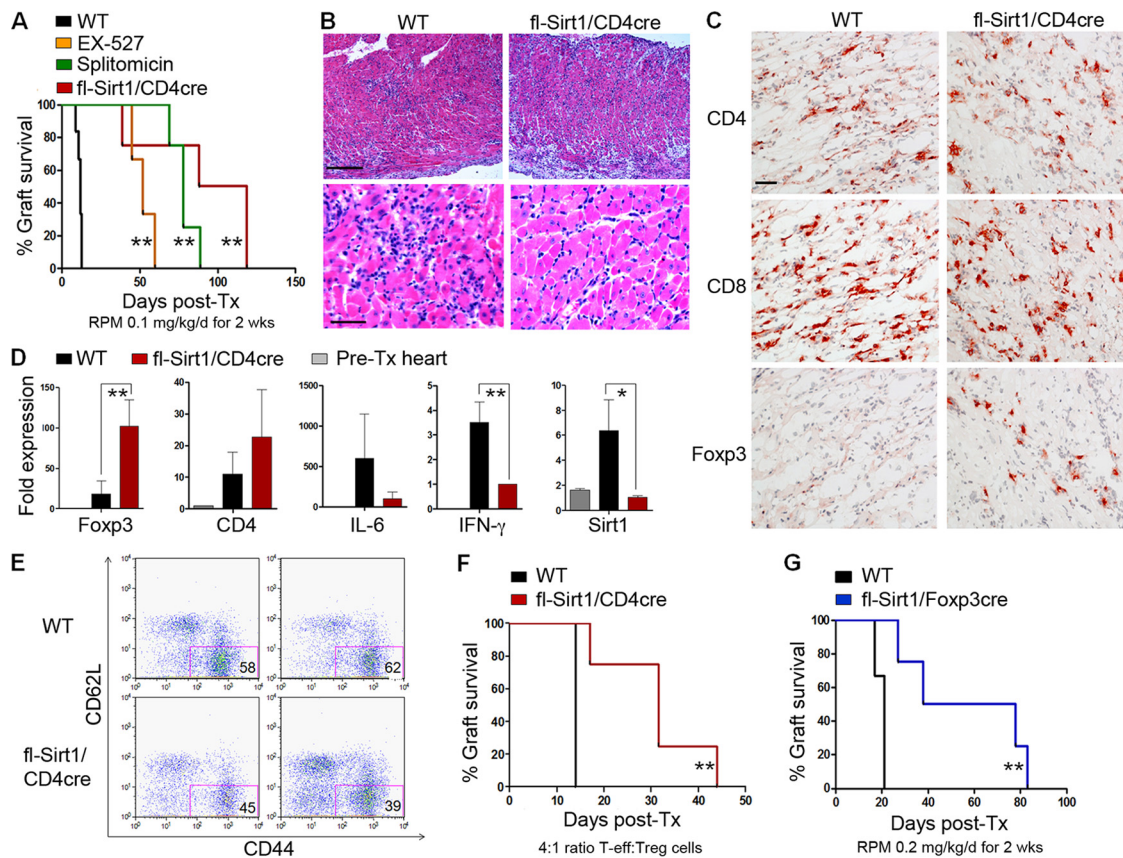


FIG. 7. Sirt1 and allograft survival. (A) Kaplan-Meier survival curves showing prolonged survival of MHC-mismatched cardiac allografts following Sirt1 deletion in CD4<sup>+</sup> T cells (fl-Sirt1/CD4cre) or Sirt1 inhibition versus WT recipients (4 to 5 grafts/group). (B) Allograft histology at 9 days posttransplant shows a similar degree of lymphocyte infiltration but better myocyte preservation, with intact nuclei and cross striations of fl-Sirt1/CD4cre recipients. For low-power (upper) or high-power (lower) magnifications, scale bars equals 200 μm or 50 μm, respectively. (C) Corresponding immunoperoxidase detection at 9 days posttransplant of CD4<sup>+</sup>, CD8<sup>+</sup>, and Foxp3<sup>+</sup> T cells in cardiac allografts in WT mice or mice with deletion of Sirt1 using CD4cre; considerably more infiltrating Foxp3<sup>+</sup> cells are noted in interstitial areas of fl-Sirt1/CD4cre versus WT recipients; scale bar = 50 μm. (D) qPCR analysis of whole-tissue RNA obtained from allografts and native BALB/c hearts (gray box) as a control, showing increased intragraft Foxp3 and decreased IFN-γ and Sirt1 in Sirt1<sup>-/-</sup> recipients. (E) Flow cytometric analysis of splenic CD4<sup>+</sup> T cells shows decreased activation in Sirt1<sup>-/-</sup> versus WT allograft recipients. (B to E are representative of 2 independent experiments). (F) B6/RAG1<sup>-/-</sup> cardiac allograft recipients were adoptively transferred with T-effector cells and WT versus fl-Sirt1/CD4cre Tregs; a significant benefit of Sirt1 deletion in Tregs was shown. (G) Likewise, fl-Sirt1/Foxp3cre recipients exhibited prolonged allograft survival compared to WT controls. Statistical analysis: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.005$ ; Mantel-Cox test versus results for untreated WT (A, F, and G) or Student *t* test versus results for BALB/c heart control (D).

Sirt1 deletion. We considered the possibility that Sirt1-deficient T cells might just be more likely to convert to a Treg lineage, since this is a known mechanism of peripheral tolerance (22). However, conversion rates of both Sirt1-deficient and T-effector cells treated with Sirt1 inhibitors were very similar to those of untreated WT T-effector cells. Treg function, on the other hand, was remarkably enhanced by Sirt1 targeting. Treg biology can be influenced by Sirt1 targeting on a number of levels. Van Loosdregt et al. have shown, by transfection of Sirt1 and Foxp3 into HEK 293 cancer cells, that Sirt1 can directly deacetylate Foxp3 and thus prevent its proteasomal degradation (28). Our data show that native Tregs from Sirt1<sup>-/-</sup> mice indeed express more Foxp3. We hypothesize that inhibiting or deleting Sirt1 can allow accumulation of acetylated Foxp3 that is protected from proteasomal degradation. Given our findings that Sirt1 is downregulated in activated Treg, this may be a mechanism to stabilize Foxp3 and

maintain the Treg phenotype. Interestingly, we found that sir-tuin inhibition via splitomicin was more potent at prolonging allograft survival than the more selective Sirt1 inhibitor, EX-527. These findings, as well as our data on the expression of other sirtuins in stimulated Tregs, suggest that additional sirtuins may be involved in regulating immune responses.

In addition to Foxp3 and RelA/p65 expression, we found that Sirt1 deletion produced a gene expression profile consistent with an increased Treg suppressive function. These include downregulation of the IL-6, IL-7 (CD127), and IL-17 cytokine receptors and of surface receptors such as CCR2, CCR9, ICAM2, integrin alpha-4, and Fas-L (2, 4, 18). Expression of the IL-7 receptor (CD127) is inversely related to Treg suppressive function (15). Moreover, we found that Sirt1 deletion led to upregulation of multiple genes involved in cholesterol metabolism and energy generation through the Krebs cycle and mitochondrial electron transport pathways, which

is consistent with the biologic profile of Sirt1-depleted cell metabolism (6). Interestingly, in studies of nonimmune cells, Westerheide et al. found that Sirt1 deacetylated heat shock factor protein 1 (HSF1) and stabilized HSF1 trimers, thereby preventing their disassembly and prolonging HSF1 transcriptional activity (31). This could lead to a decreased heat shock response in Sirt1<sup>-/-</sup> cells, and indeed, we found decreased expression of several HSP targets of HSF1. Clearly, further studies are required to analyze the effects of Sirt1 targeting on Treg energy metabolism and heat shock responses.

In conclusion, our data show that both Sirt1 deletion and Sirt1 inhibition cause prolonged allograft survival. This effect is mediated chiefly through enhanced Treg suppressive function, since effector T cell responses are essentially unaffected. Sirt1 targeting in Tregs promotes Foxp3 expression and alters expression of many genes, including those involved in energy metabolism. We conclude that in addition to its roles in determining the caloric restriction response and cellular longevity, Sirt1 may regulate Treg functions, such that its targeting may have therapeutic value in autoimmunity and transplantation.

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